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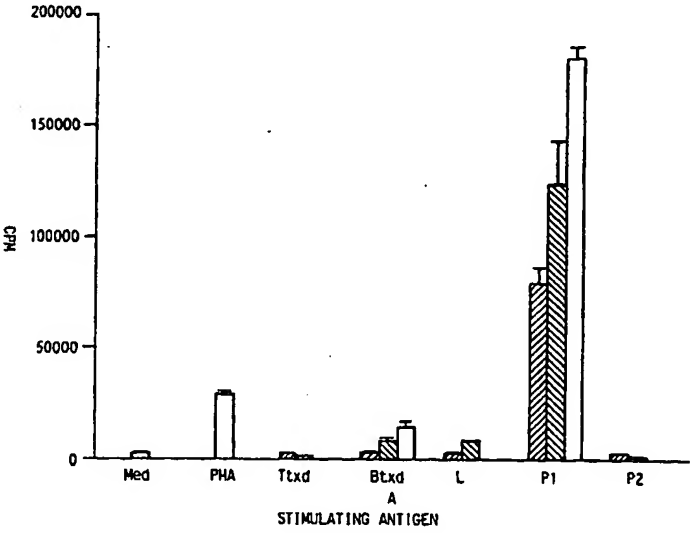
H_N fragments



(57) Abstract

A short polypeptide, typically in the range 7-35 amino acid residues, that contains a Botulinum toxin T cell epitope and is useful for administration to a mammal as part of a vaccination therapy. Following administration of the polypeptide, an individual has an enhanced response to known botulinum toxin vaccines, such as toxoid vaccine; thus the effects of the vaccine can be improved and increased. Polypeptide formulations and vaccination methods are described.

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BOTULINUM TOXIN POLYPEPTIDES AND THEIR USE AS VACCINE ENHANCING AGENTS

This invention relates to polypeptides useful as vaccine enhancing agents for use in vaccinating a patient against botulism. The invention also relates to vaccines incorporating the polypeptides, to methods of vaccinating a patient against botulism using the polypeptides in combination with vaccines and to kits incorporating the polypeptides and optionally containing vaccines.

Clostridium botulinum are a group of seven anaerobic bacterial strains that produce antigenically distinct neurotoxins designated type A, B, C_1, D, E, F and G which are extremely powerful presynaptically acting neuroparalytic agents lethal to animals and man. It has been demonstrated that types A, B, E and F botulinum neurotoxin cause human disease, both food-borne and infant botulism. For more than 40 years, immunisation with botulinum toxoid has been used to protect laboratory workers at risk from botulism due to contact with the neurotoxins.

A problem with a known toxoid vaccine preparation is that it is relatively crude and produces undesirable local and systemic reactions. Also the current pentavalent (ABCDE) toxoids manufactured by the Michigan Department of Public Health induce significantly improved response to type B but not to type A and E toxin. Furthermore, a long period, typically 12-25 weeks is required to achieve a protective level and the range of antibody titres among individuals who received the same number of immunisations is very wide.

The development of alternative vaccines, vaccine components and methods for vaccinating patients against disease caused by botulinum toxins is desirable.

The development of vaccination methods that reduce the number of immunisations required using the known, e.g. pentavalent, toxoid, is desirable as this might reduce the undesirable reactions.

The invention thus addresses the problem, generally, of providing improved vaccination against botulism.

It is an object of the present invention to overcome and/or at least mitigate some of the disadvantages in known vaccines and methods of vaccination.

The present invention offers a solution to the problems encountered with current vaccines in the form of a polypeptide which is free of botulinum toxin activity but which contains a T cell epitope and can stimulate, in response to subsequent administration of botulinum toxoid, T cell proliferation and an enhanced production of anti-botulinum toxin antibodies in a patient. The present invention also offers a solution to the problems inherent in existing vaccination methods in the form of a method that includes administration of the polypeptide of the invention in combination with known vaccines.

According to a first aspect of the invention, there is provided a polypeptide, substantially free of botulinum toxin activity, which is capable of priming a population of T cells in a mammal to proliferate in response to a botulinum toxoid. By "priming" it is intended to mean that initial exposure to the polypeptide has the effect that the reaction to subsequent exposure to a botulinum toxoid is increased, compared to the reaction to a botulinum toxoid in the absence of initial exposure to the polypeptide.

In a second aspect, a polypeptide of the invention comprises a T cell epitope and is capable of priming a population of T cells to proliferate in response to subsequent exposure to a contact with botulinum toxoid; the polypeptide being substantially free of toxin activity.

As as example of the polypeptide in use, the polypeptide is adminstered to a human to protect against the danger of disease caused by botulinum toxin. The polypeptide's effect is to make the immune system of that person more sensitive to botulinum toxoid. That person is subsequently given a dose of botulinum toxoid. The increased sensitivity results in increased formation of antibodies against the toxoid that protect against botulinum toxin.

The polypeptides of the invention are thus of particular advantage in that they can enhance or increase the effectiveness of existing toxoid vaccines. In addition, vaccination aided by using the polypeptides may be achieved in a shorter time than previously possible.

The invention is based on the discovery that a botulinum toxin molecule contains a region which, in isolation from the rest of the toxin, can be used to sensitize a patient against subsequent contact with that same region. The invention utilizes the discovery to provide a concentrated, non-toxic, source of the sensitizing region that acts to enhance the properties of botulinum toxoid vaccines.

In an embodiement of the invention, a polypeptide comprises a sequence of 35 amino acids having a degree of homology of at least 50% with a corresponding sequence of at least one sub-type of botulinum toxin and is free of botulinum toxin activity, or comprises a sub-fragment thereof at least 7 amino acids in length. The polypeptide fragment is preferably up to 25 amino acids in length, and most preferably up to 18 amino acids in length.

As indicated, the polypeptides provided according to the invention can comprise a specific sequence of 7 to 35 amino acids. Thus they may consist solely of such a sequence or they may consist of the specified sequence of from 7 to 35 amino acids conjugated or otherwise linked to other sequences. Clearly such other sequences should not be such that the polypeptide as a whole possesses botulinum toxin activity.

In a particularly preferred embodiment of the invention the polypeptide consists of the sequence of from 7 to 35 amino acids i.e. the polypeptides do not include sequences in addition to the specified sequence.

The degree of homology with the corresponding sequence of botulinum toxin is, in an embodiment of the invention, at least 75% and in a preferred embodiment at least 90%. In a further preferred embodiment the homology is at least 95%.

Polypeptides according to the first and second aspects of the invention are exemplified by a polypeptide comprising the amino acid sequence of SEQ ID No:1, or an N-terminal fragment of said amino acid sequence of at least 7 amino acid residues in length. A particularly preferred embodiment consists of the 7 N-terminal amino acids of SEQ ID NO:1. The invention further includes such amino acid sequences comprising up to three additions, substitutions or deletions, provided the resulting polypeptide is at least 7 amino acids long.

In such polypeptides according to the invention it is particularly preferred that not more than two amino acids are substituted, added or deleted and very preferably, 0 or 1 amino acids are substituted, added or deleted.

Substitutions to the polypeptide sequence of SEQ ID No:1 can substitute any amino acid for any other but are preferably made according to the following table:-

Original Amino Acid	Optional Substitute
Arg	His, Lys
Pro	Ala
Ala	Pro
Val	Met, Ile, Ala, Phe
Ile	Met, Leu, Phe, Val
Phe	Met, Tyr, Ile, Leu
His	Gln, Arg, Lys
Gln	Glu, His
Asn	Asp, Gln
Lys	Arg, His
Asp	Asn, Glu
Glu	Gln, Asp
Met	Ile, Leu, Phe, Val

Polypeptides according to the invention and based on SEQ ID NO:1 are preferably up to 30 amino acids in length.

According to a third aspect of the invention there is provided a vaccine enhancing agent for use in vaccinating a patient against botulism comprising a polypeptide according to any of the first or second aspects of the invention in combination with a suitable pharmaceutical excipient or carrier.

In one embodiment of the third aspect of the invention the vaccine enhancing agent additionally comprises a known botulinum toxoid vaccine such as, for example, pentavalent (A,B,C,D,E) toxoid vaccine.

According to a fourth aspect of the invention there is provided a method of vaccinating a mammal against botulism comprising the steps of:

(1) administering to the mammal a first polypeptide according to the invention, and

(2) subsequently or simultaneously administering to the mammal a second vaccine comprising a known botulinum toxoid vaccine.

An alternative method according to the fourth aspect of the invention is to first administer the known botulinum toxoid vaccine and subsequently to administer a polypeptide according to the third aspect of the invention.

In a fifth aspect of the invention there is provided a kit for vaccinating a patient against botulism comprising supplies of a polypeptide according to the invention together with supplies of a known botulinum toxoid vaccine.

According to a sixth aspect of the invention there is provided a method of vaccinating a mammal against a disease caused by a polypeptide toxin comprising the steps of:

(1) administering to the patient a vaccine enhancing agent comprising a polypeptide, substantially free of toxin activity, which contains a T cell epitope and

is capable of increasing the anti-toxin antibody titre of a patient subsequently challenged with the toxin or with chemically de-activated toxin, and

(2) subsequently administering toxin or chemically de-activated toxin to the patient.

The polypeptides according to the invention are of advantage in that they can readily be synthesised and are thereby available to a high degree of purity for use in vaccinating a mammal against botulism.

Since botulinum toxin has a molecular weight of approximately 150,000 daltons, the polypeptides of the invention, which are relatively very short molecules and free of botulinum toxin activity, will not cause botulism in the vaccinated subject.

There now follows a description of exemplary embodiments of the invention in which:

Fig. 1 shows an ELISA antibody response to botulinum toxoid, and

Fig. 2 shows an ELISA antibody response to toxin.

The antibody titres were assayed from sera obtained from three groups of mice immunised with botulinum toxoid only (square); with toxoid and alum (circle); and with alum only (triangle).

Fig. 3 shows proliferative T cell response of mice immunized with P1, a polypeptide of the invention. Values are mean ±1 SEM, N=3. The stimulating antigens used were med, medium control; PHA, phytohaemagglutinin; TTxd, tetanus toxoid; Btxd A, botulinum toxoid A; L, light chain Btxd A. The antigen concentrations used for stimulation were 2μg/ml (stripes, bottom left to top right), 10μg/ml (stripes, top left to bottom right) and 50μg/ml (open box).

Fig. 4 shows the effect of priming with P1 on antibody response.
The results show response after 10 days following immunisation with Btxd A, pre-primed with adjuvant control (open box) and the same after 21 days (stripes top right to bottom left) and response after 10 days following the same immunisation, pre-primed with P1 (stripes, top left to bottom right) and then after 21 days (stripes horizontal).

Example 1

Three human donors immunised with a pentavalent botulinum toxoid (A-E) vaccine showed strong proliferative T cell response to toxoid A and to the synthetically cleaved fragments L, H, and L-H_N of botulinum toxin A (BoNT/A). A 27-mer peptide (aa 29-54 designated SEQ ID No: 1) in the N-terminal region of the toxin was also recognised by the T cells of two of the three donors. The importance of such T cell response was further investigated in mice immunised with toxoid A in the presence or absence of alum. The murine T cells also recognised the toxoid, BoNT/A fragments and peptide aa 29-54. Such proliferate T cell response is augmented by the presence of alum as adjuvant. Antibodies taken from several mice immunised with toxoid A were able to neutralise botulinum toxin A *in vitro* and protect mice against neurotoxicity *in vivo* on subsequent injection. The protective antibody titre was 7 fold higher in the group given toxoid with alum than without. Although there was a 10 fold increase in ELISA antitoxin antibody response in the group given toxoid with alum compared with that without alum, no difference in ELISA antibody titre to the toxoid was detected between the groups, suggesting that just antitoxin antibody response is augmented. Therefore, the co-induction of higher (7-10 fold) protective antitoxin response with T cell response suggests that effective protection requires the co-induction of a strong T and B cell response.

Materials and methods

Mice Female BALB/c mice between 8-10 weeks old were bred at NIBSC. Female MF1 mice weighing between 18-20gm were obtained from Harland Olac (Bicester, Oxon, UK).

Immunisations BALB/c mice were immunised subcutaneously in the flank and footpad with 10μg botulinum toxoid type A per mouse. Where aluminium hydroxide was used as adjuvant, a 1:10 dilution of Alhydrogel (Superfos Vedbaek, Sweden) was mixed and incubated with toxoid in phosphate buffered saline overnight at 4°C before use. The animals were given booster immunisations 2 weeks later followed by a second booster 4 weeks after that.

Preparation of botulinum toxoid and toxin fragments H, L, and L-H_N

Botulinum toxin type A was purified from 20 litres of 48hr culture supernatant of *Clostridium botulinum* type A (A 7272) by acid precipitation followed by affinity chromatography (as described by Shone in Eur. J. Biochem 151:75–82). The neurotoxin so obtained had a specific activity of 1.5 – 2 x 10^8 LD_{50}/mg. The toxin was detoxicated by incubating with 0.2% formaldehyde for 25 days at 37°C. The resultant toxoid was extensively dialysed against HEPES buffer (0.05M, pH 7.4) containing 0.2M NaCl to remove excess formaldehyde before use. Protein concentration was determined by the method of Bradford (in Anal. Biochem. 72:248–254), using crystalline bovine serum albumin (fraction V, SIGMA) as standard.

The heavy (H) and light (L) chain fragments of botulinum toxin (BoNT/A) were prepared as previously described with some modifications. Briefly, 0.5mg purified botulinum toxin in borate/phosphate buffer (0.05M, pH 8.5) was loaded on to a 3 ml QAE Sephadex A50 (Pharmacia) column previously equilibrated with the same buffer and the column washed with approximately 20 ml of borate/phosphate buffer containing 10mM dithiothreitol (DTT). Then a volume of 100mM DTT and 2M urea in borate/phosphate buffer, equivalent to the void volume of the column, was applied to the column and the whole incubated overnight at 4°C. Fragment L was eluted at 4°C with 10mM DTT, 2M urea in borate/phosphate buffer. Fragment H was subsequently eluted with 0.2M sodium chloride in borate/phosphate buffer. Both protein fragments were separately collected and dialysed extensively in HEPES buffer (0.05M, pH 7.4) containing 0.15M sodium chloride.

The 105kDa L-H_N fragment of botulinum toxin was prepared essentially as previously described. Briefly, the purified toxin was treated with 50μg/ml

tosylphenylalaninechloromethane-treated trypsin in phosphate saline buffer (0.15M NaCl, 0.05M sodium phosphate pH 8.0) at 20°C for 72hr. The digest was chromatographed on a column consisting of 2ml α_2-macroglobulin-agarose layered onto 1 ml trypsin-inhibitor-agarose phosphate/saline buffer. Major fractions eluted with phosphate/saline buffer were pooled, concentrated and re-chromatographed on a Sephadex G200 (Pharmacia) column pre-equilibrated with the same buffer. Fractions from the first protein peak containing L-H_N were collected and pooled.

ELISA assay The ELISA assay was performed as previously described (by Voller in Bull. World Health Organisation 53:55-65). Briefly, 100µl/well of botulinum toxoid or toxin at 1µg/ml in 0.1M carbonate buffer pH 9.6 were used to coat 96-well microtitre plates (Nunc, maxi sorb) overnight at 4°C. Sera at the indicated dilutions and rabbit anti-mouse Ig conjugated with peroxidase (1:5000, SIGMA) were diluted in PBS containing and 0.05% Tween 20. All washes were with PBS containing 0.1% skimmed milk powder and 0.05% Tween 20. The enzyme substrate used was 2,2'-Azino-bis(3-ethyl -benzthiazoline-6-sulfonic acid (SIGMA)) with hydrogen peroxide and O.D. readings were taken at 405nm with an Anthos ELISA plate reader model 2001 (Anthos Labtec Instruments, Austria).

Synthesis of peptides Peptides were synthesised using an automated, solid-phase peptide synthesizer (model 431A, Applied Biosystems Inc., Foster City, CA.) using Applied Biosystems' FastMoc™ chemistry. Peptides were synthesised on either preloaded p-benyloxybenzyl alcohol resins or on 4-(2',4'-dimethoxyphenyl Fmoc-aminomethyl)-phenoxy resin (Novabiochem (UK) Ltd.) and the purity checked by HPLC analysis.

Proliferations assay All assays were set up in triplicates in 96-well flat-bottomed tissue culture plates as previously described (by Chan in Immunology. 68: 96-101). Draining lymph nodes were obtained from mice 7 days after the second boost. Lymph node cell suspensions were dispensed at 4 x 10^5 cells per well in 100µl of Click's Eagle's high amino acid medium supplemented with 2 mM L-glutamine, 2 x 10^{-5}M 2-ME and 0.5% normal mouse serum. The human donors were healthy males and females vaccinated with

a pentavalent botulinum toxoid vaccine. Human unfractionated mononuclear cells were obtained from peripheral blood by gradient centrifugation through lymphoprep (Nyegaard and Co., Oslo, Norway) after defribrination with a bent glass rod and dispensed at 2 x 10^5 cells per well in 100µl of RPMI-1640] medium supplemented with 2mM L-glutamine, 2 x 10^{-5}M 2-ME and 12.5% autologous serum. Each culture, with or without antigen, was incubated in an atmosphere of 5% CO_2 at 37°C for 4 days with mouse cells of 5 days in the case of human cells, pulsed during the last 18hr with 1µCi per well of [^{3}H] thymidine (Amersham, Bucks) and harvested with a cell harvester.

in vivo botulinum toxin neutralisation assay The potency of botulinum antitoxin in the sera of mice immunised with the toxoid A was determined by titration against a standard preparation of botulinum antitoxin (WHO International Standard for type A antitoxin, 500 IU/ampoule at IU=0.136 mg protein) to give the same degree of protection with a fixed dose of botulinum toxin ($50LD_{50}$). Equal volume of sera from each of the three mice of each group were pooled and diluted 1:100 in gelatin phosphate buffer (0.05M sodium phosphate buffer pH 6.5 containing 0.2% gelatin). Then graded volumes of the diluted sera or reference antisera (0.5IU/ml) were each mixed with a fixed concentration of botulinum toxin (calculated to deliver a dose of $50LD_{50}$/mouse) to a final volume of 4.5ml per antibody dilution per group of 4 mice. The mixture was incubated at room temperature for 1 hr before 1ml or it was injected intraperitoneally per MF1 mouse. The animals were observed for signs of neurotoxicity over a period of 4 days. The assay for potency of the human sera samples was essentially the same except that the dose of botulinum toxin A was $10LD_{50}$ per mouse and reference antisera concentration use was 0.1U/ml.

The following results were obtained.

Proliferative T cell response in human vaccinated with botulinum toxoid. Three individuals were vaccinated 2 (VK) or 3 times (DS and MJC) with a pentavalent botulinum toxoid vaccine before they were tested in presence of stimulating antigen for proliferative T cell response *in vitro*. As shown in Table 2, cells from all 3 individuals responded well to botulinum toxoid (BTxd) and BoNT/A fragments H and L; those of

MJC showing a much stronger response than those of the other two. None of them responded to control protein bovine serum albumin (BSA). However, when 5 peptides, each 27 mer long and synthesized from the amino acid sequence of fragments L (peptide 1 and 2) (SEQ 1D NO: 1, SEQ ID NO: 2) or H (peptides 3–5) (SEQ ID NO: 3–SEQ ID NO: 5) of the toxin were used as stimulating antigens, peptide 1 (spanning aa 28–53 of botulinum toxin A) was recognised by T cells from 2 of the individuals. The proliferative T cell response from VK to peptide 1 was very strong but a much weaker response was detected with cells from DS despite the fact that VK had 1 immunisation less than DS. The T cells of MJC recognised peptide 4 and to a lesser extent peptide 3.

Proliferative T cell response in mice BALB/c mice immunised with botulinum toxoid type A in the presence of alum as adjuvant showed stronger *in vitro* proliferative T cell response to botulinum toxoid (BTxd), BoNT/A fragments L, H, and L–H_N than those immunised without alum (Table 3). Cells from control mice given alum alone did not respond to any of the antigens *in vitro*. In addition, like the human T cells, the murine T cells also recognised peptide 1 (aa 29–54); the response being markedly stronger in mice immunised with toxoid in the presence than in the absence of alum.

ELISA antibody response to toxoid and to toxin There was no significant difference in the ELISA antibody titre to botulinum toxoid, L and H of sera from mice immunised with toxoid in the presence or absence of alum (Fig. 1). However when tested on toxin, the ELISA antibody titre was 10 fold higher in the group given toxoid with alum (Fig. 2).

Protective antibody response to toxin Sera from groups of mice immunised with toxoid A in the presence or absence of alum contain protective antitoxin antibodies that were able to neutralise the toxin. Significantly, mice from the group that was immunised with toxoid in the presence of alum had 7 times higher level of antitoxin antibody response than those not given alum (Table 4).

Levels of protective antitoxin antibodies were also measured in human sera. VK had the highest neutralising antitoxin antibody titre even though she had received only 2 vaccinations while DS had the lowest titre despite 3 vaccinations (Table 4).

Table 1

The amino acid sequence and position of the peptides on botulinum toxin type A

Peptide	Amino acid sequence	Position	SEQ ID
P1	GQMQPVKAFKIHNKIWVIPERDTFTNC	L 28-53	NO: 1
P2	ATDPAVTLAHELIHAGHRLYGIAINC	L 214-238	NO: 2
P3	CVNNTLSTDIPFQLSK	H 841-855	NO: 3
P4	CVDNQRLLSTFTEYIK	H 857-871	NO: 4
P5	DVNNVGIRGYMYLKGPRGSC	H1124-1142	NO: 5

* The C-terminal Cys in P1, P2 and P5 and the N-terminal Cys in P3 and P4 are not part of the toxin sequence.

\+ The numbering of peptide position is from the C-terminal Met on the botulinum toxin type A aa sequence deduced from the cDNA nucleotide sequence (Thompson et al.,1990).

Table 2

Proliferative T Cell Response in human vaccines

Stimulating Antigen* (µg/ml)		[^{3}H]TdR incorporation (CPM)+ VK	DS	MJC
Med		486 ± 13	297 ± 73	590 ± 59
PHA		332213 ± 12212	71161 ± 3577	127973 ± 8181
BSA	(5)	453 ± 27	289 ± 52	400 ± 33
BTxd	(5)	64042 ± 14834	60395 ± 3652	259572 ± 19086
	(1)	39538 ± 4428	50724 ± 1775	ND
L	(5)	65830 ± 14521	53928 ± 5995	178867 ± 18003
	(1)	33638 ± 6219	26782 ± 1900	95035 ± 8030
H	(5)	38400 ± 2009	47352 ± 10304	149619 ± 3331
	(1)	14353 ± 4430	20908 ± 7857	60213 ± 1701
L-H_N	(5)	75656 ± 15980	67961 ± 7784	ND
	(1)	32833 ± 4432	8186 ± 3771	ND
P1	(25)	32062 ± 4778	2458 ± 1469	650 ± 62
	(2.5)	14068 ± 6058	1187 ± 851	647 ± 53
	(0.25)	13844 ± 5893	301 ± 40	356 ± 16
P2	(25)	486 ± 127	751 ± 279	1894 ± 1166
	(2.5)	1019 ± 389	384 ± 205	603 ± 144
P3	(25)	811 ± 69	554 ± 139	2102 ± 407
	(2.5)	491 ± 37	256 ± 58	441 ± 60
P4	(2.5)	867 ± 101	315 ± 57	2786 ± 452
	(0.25)	1344 ± 392	677 ± 160	3178 ± 452
P5	(25)	655 ± 116	331 ± 39	598 ± 61
	(2.5)	217 ± 44	184 ± 23	434 ± 53

* The stimulating antigens used were Med, medium control; PHA, phytohemagglutinin; TTxd, tetanus toxoid; BTxd, botulinum toxoid; P1-5, peptides 1-5 as in table 1.

+ Each value represents mean ± 1 SEM, N=3. ND represents values not determined.

Table 3

Proliferative T Cell Response in mice

Stimulating Antigen* (µg/ml)		[^{3}H]TdR incorporation (CPM)+ BTxd	Alum + BTxd	Alum
Med		270 ± 48	372 ± 61	213 ± 14
PHA		32530 ± 760	38647 ± 1035	42654 ± 6604
TTxd	(5)	149 ± 6	178 ± 9	185 ± 28
BTxd	(5)	1168 ± 106	25195 ± 1280	253 ± 18
	(1)	ND	13011 ± 1635	ND
L	(5)	1037 ± 261	7629 ± 630	237 ± 26
	(1)	460 ± 83	3561 ± 1208	ND
H	(5)	1271 ± 88	20216 ± 6443	160 ± 39
	(1)	298 ± 90	10547 ± 1892	ND
L-H_N	(5)	552 ± 178	17021 ± 2710	165 ± 28
	(1)	284 ± 42	4185 ± 146	ND
P1	(25)	736 ± 203	3435 ± 759	189 ± 42
	(2.5)	363 ± 104	3405 ± 366	ND
	(0.25)	ND	576 ± 60	ND
P2	(25)	320 ± 23	361 ± 32	161 ± 12
P3	(25)	391 ± 53	509 ± 102	212 ± 30
P4	(25)	355 ± 25	593 ± 55	184 ± 42
P5	(25)	209 ± 38	197 ± 18	93 ± 2
P6	(5)	244 ± 5	254 ± 63	233 ± 23

* - as Table 2

+ - as Table 2

Table 4

Neutralising effect of antisera from murine and human vaccinees on the *in vivo* toxicity of BoNT/A

Vaccinee	Potency (IU/ml)
Mice	
BTxd + Alum	690
BTxd alone	100
Alum alone	<0.2
Human	
VK	2.3
DS	0.28
MJC	0.87

Reference antisera used has potency of 0.5 IU/m

Example 2

Purification of *Clostridium botulinum* neurotoxin A *Clostridium botulinum* type A (strain 7272) was grown in 20–l batches on USA 2 growth medium, and the neurotoxin purified from a total of 200 l bacterial culture by a modification of the method described for botulinum type–F neurotoxin. For the final chromatography stage, neurotoxin was dialysed against 101, 0.05 M sodium succinate, pH 5.5, and loaded onto a column (2.6 cm x 6 cm) of Sepharose S (Pharmacia) and eluted with a 200–ml linear NaCl gradient (0–0.3 M) in the succinate buffer. BoNTA, the major protein peak to elute during the NaCl gradient, was dialysed against 0.05 M Hepes, pH 7.4 containing 0.15 M NaCl (Hepes/NaCl), aliquoted and stored at –80°C.

Example 3

Purification of LH_N fragment of type A neurotoxin. Botulinum neurotoxin (40 mg in 15 ml) in 0.15 M Tris/HCl buffer, pH 8.0 containing 100 mM NaCl was treated with trypsin (50 μg ml^{-1}) for 72–96 h at 20°C, dialysed against 20 mM triethanolamine buffer, pH 7.8 containing 100 mM NaCl, filtered (0.22 μm pore size) and then chromatographed (fast protein liquid chromatography system, Pharmacia) on a Mono Q (HR 10/10, Pharmacia) equilibrated in the latter buffer. The column was washed with a further 100 ml of the triethanolamine buffer and then the H_2L fragment eluted with triethanolamine buffer (20 mM, pH 7.8) containing 200 mM NaCl. The purified fragment (≈1.5 mg ml^{-1}) was made 5 μg ml^{-1} with trypsin inhibitor, dialysed against 0.15 M Tris/HCl buffer, pH 8.0 containing 5 mM NaCl and stored at –20°C.

Example 4

In this study, when we immunised mice with botulinum toxoid A, enhanced neutralising antibody response to toxin A was elicited only in mice that had been pre–primed with a 27–mer toxin A peptide according to the invention which had induced a proliferative T cell response. The results show that priming at the T cell level using a peptide of the invention provides sufficient help to drive an accelerated antibody response and thereby reduce the immunisation period and unnecessary exposure to the adverse side effects of the toxoid vaccine.

MATERIALS AND METHODS

Mice – as Example 1.

Immunisations BALB/c mice were immunised subcutaneously in the flank with 50μg P1 per mouse in the presence of Freund's complete adjuvant. The animals were given a booster immunisation of antigen in phosphate buffered saline subcutaneously.

Preparation of botulinum toxoid and toxin fragments H and L – as Example 1.

Radioimmunoassay The RIA was preformed as previously described in J. Exp. Med. 162:1304. Briefly, 100µl/well of botulinum toxoid or toxin at 1µg/ml in 0.1M carbonate buffer pH 9.6 were used to coat flexible 96-well microtitre plates (Falcon) overnight at 4°C. Sera at the indicated dilutions and rabbit anti-mouse Ig labelled with ^{125}I were diluted in PBS containing 10% Newborn calf serum and 0.05% Tween 20. All washes were with PBS containing 0.05% Tween 20. The wells were cut out and radioactivity counted in a gamma-counter.

Synthesis of peptides - as Example 1

Proliferation assay All assays were set up in triplicates in 96-well flat-bottomed tissue culture plates as previously described. Draining lymph nodes were obtained from mice 7-14 days after the first boost. Lymph node cell suspensions were dispensed at 4×10^5 cells per well in 100µl of Click's Eagle's high amino acid medium supplemented with 2 mM L-glutamine, 2×10^{-5} M 2-ME and 0.5% normal mouse serum. Each culture, with or without antigen, was incubated in an atmosphere of 5% CO_2 at 37° for 4 days, pulsed during the last 18 hr with 1µCi per well o [^3H]thymidine (Amersham, Bucks) and harvested with a cell harvester.

Cell proliferation is expressed as SI [Stimulation index = (c.p.m. with antigen)/(c.p.m. without antigen)]. SI values greater than 2.5 are considered significant.

in vivo botulinum toxin neutralisation assay - as Example 1

RESULTS

Proliferative T cell response of mice immunised with P1 Draining Lymph node cells from 3 mice immunised with P1 in the presence of Freunds complete adjuvant were tested for the presence of proliferative T cell response *in vitro*. As shown in Fig. 3, the cells responded well to BTxd A, L and to P1. The response to P1 was very strong with SI = 74 - 33 when peptide concentrations of 50 - 2µg/ml were used for stimulation. The response to BTxd A and L were about 10 fold lower for the same concentration range of

the two antigens used. There were no response to control peptide 2 (P2, aa 214–238) and to tetanus toxoid (TTXd).

Priming with peptide 1 augments RIA antibody titre to H and Btxd A When mice were preprimed with P1 in the presence of Freund's complete adjuvant before immunisation with BTxd A, the RIA antibody titre of sera taken at 10 and 21 days later to H, and BTxd A were 4–10 fold higher than that of control mice given Freund's complete adjuvant alone followed by BTxd A (Fig 4).

Accelerated Antitoxin antibody response in mice primed with peptide P1 Sera from mice immunised with BTxd A after prepriming with Freund's Complete adjuvant in the presence or absence of P1 contain protective antitoxin antibodies that were able to neutralise botulinum toxin A. Significantly, at 21 days post immunisation with BTxd A, mice from the group that were preprimed with P1 had 10 fold higher level of antitoxin antibody response than those not given the peptide (Table 5).

Helper T cell epitope is present in homologous regions of toxoid A, B and E When draining lymph node cells from mice primed with P1 were stimulated *in vitro* with BTxd A, B, C, E, or F there was strong proliferative T cell response to BTxd A, B and F and a very weak response to BTxd C. The T cells did not respond to BTxd E. Further mapping of the Th epitope using 3 peptides overlapping by 10–mer shows that it is located within aa 29–43 (Table 6).

Thus, priming with P1 results in an augmented antibody response induced by BTxd A. The cross reactive T cell response induced by P1 to BTxd B and F and to a limited extent to C suggests that immunising with P1 is of potential use in augmenting the anti–botulinum toxin B, C and F responses as well as the anti–A reponse. Therefore immunising with a peptide of the invention which contains a Th epitope prior to immunisation with a different vaccine, such as a toxoid vaccine exemplified by BTxd A, augments the anti–toxin antibody response. Such a regime helps to boost the immune response to the toxoid vaccine, thus reducing the duration and number of immunisations and also side effects from exposure to the vaccine.

Table 5

Accelerated antitoxin antibody response in mice primed with P1

Immunisation		Potency (IU/ml)
Primary	Boost	
CFA + P1	BTxd A	3.01
CFA alone	BTxd A	0.27
None	None	<0.05

The adjuvant used for priming was Complete Freund's Adjuvant (CFA).

As will be appreciated from the results herein, the present invention provides vaccine enhancing agents for therapeutic use in vaccinating against disease caused by botulinum toxin as well as providing improved methods for vaccinating a subject against disease caused by a polypeptide toxin, such as botulinum toxin.

The examples show how relatively small polypeptides of even only up to 27 amino acids in length can provide the basis for a vaccine enhancing agent for generating immunity against a toxin molecule of molecular weight approximately 150,000 daltons. The particular amino acid sequence that is effective in the example is not the only region of the whole toxin polypeptide that is immunogenic and so the invention also relates to other polypeptide regions of the toxin molecule that are immunogenic and which, when used in isolation from the rest of the toxin are effective in stimulating the production of anti-toxin antibodies but are substantially free of toxin activity.

Example 5

A combined peptide and botulinum toxoid vaccine.

A vaccine consisting of a quantity of the peptide of the invention (e.g. in the range 0.1-50 μg ml) plus a quantity of botulinum toxoid (e.g. in the range 0.1 - 20 μg/ml) absorbed

onto suitable carrier e.g. alhydrogel (alum) (not greater 0.15mg/ml aluminium, final concentration) in the presence of a preservative e.g. thimerisol (not more than 0.01% final concentration). Administration of one or more doses (0.5-1ml) is by various routes (e.g. intra-muscularly, sub-cutaneously) at various intervals. A vaccine can also be created by blending the absorbed peptide of the invention with existing botulinum vaccines.

Table 6

Helper T cell epitope maps to homologous regions of BTxd A, B, C and F

Stimulating Antigen (µg/ml)		[^{3}H]TdR incorporation** (CPM)	Stimulating Index
Expt.1			
Med		436 ± 101	-
P1	(5)	12948 ± 995	30
P2	(5)	227 ± 49	0.5
B5	(25)	4577 ± 383	11
	(5)	4099 ± 473	9.4
B6	(25)	2719 ± 615	6.2
B7	(5)	386 ± 70	0.9
Expt. 2			
Med		246 ± 101	-
TTxd	(0.1)	284 ± 57	1.2
BTxd A	(25)	5497 ± 370	22.3
	(5)	1663 ± 162	6.7
BTxd B	(25)	2070 ± 230	8.4
	(5)	816 ± 139	3.3
BTxd C	(5)	1148 ± 137	4.7
BTxd E	(25)	88 ± 12	0.4
	(5)	129 ± 5	0.5
BTxd F	(25)	1985 ± 49	8.0

*Stimulating antigen use are Med, medium control; P1, P2, B5, B6 and B7 are BTxd A peptides with amino acid sequences of 29–54, 214–238, 29–43, 34–46 and 40–54 respectively.

**Each value represents means ± 1 SEM, N=3.

Example 6

A two component vaccine

Component 1 consists of the peptide of the invention absorbed onto a suitable carrier with or without preservative as defined above in Example 5. Component 2 consists of a known botulinum vaccine. Administration of one or more doses of the component 1 is followed after an interval (e.g. 7–14 days) by one or more doses of component 2.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(A) NAME: Woon Ling CHAN
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(E) COUNTRY: UK
(F) POSTAL CODE (ZIP): EN6 3QG

(A) NAME: Dorothea SESARDIC
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(C) CITY: Potters Bar
(D) STATE: Hertfordshire
(E) COUNTRY: UK
(F) POSTAL CODE (ZIP): EN6 3QG

(ii) TITLE OF INVENTION: BOTULINUM TOXIN POLYPEPTIDES AND THEIR USE AS VACCINE ENHANCING AGENTS

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Clostridium botulinum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
Gly Gln Met Gln Pro Val Lys Ala Phe Lys Ile His Asn Lys Ile Trp
1 5 10 15
Val Ile Pro Glu Arg Asp Thr Phe Thr Asn Cys
20 25

(2) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Clostridium botulinum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
Ala Thr Asp Pro Ala Val Thr Leu Ala His Glu Leu Ile His Ala Gly
1 5 10 15
His Arg Leu Tyr Gly Ile Ala Ile Asn Cys
20 25

(2) INFORMATION FOR SEQ ID NO: 3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Clostridium botulinum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Cys Val Asn Asn Thr Leu Ser Thr Asp Ile Pro Phe Gln Leu Ser Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Clostridium botulinum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Cys Val Asp Asn Gln Arg Leu Leu Ser Thr Phe Thr Glu Tyr Ile Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Clostridium botulinum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu Lys Gly Pro
1 5 10 15
Arg Gly Ser Cys
20

CLAIMS

1. A polypeptide substantially free of botulinum toxin activity and capable of priming a T cell to proliferate in response to a botulinum toxoid.

2. A polypeptide as claimed in Claim 1, comprising a botulinum toxin T cell epitope.

3. A polypeptide as claimed in Claim 1 or 2 additionally capable of stimulating the production of antibodies in response to subsequent administration of botulinum toxoid.

4. A polypeptide as claimed in any preceeding claim comprising a sequence of from 7 to 35 amino acids having a degree of homology of at least 50% with a corresponding sequence of at least one sub-type of botulinum toxin, and being substantially free of botulinum toxin activity.

5. A polypeptide as claimed in Claim 4 comprising up to 25 amino acids of said sequence.

6. A polypeptide as claimed in Claim 4 or 5 in which the degree of homology is at least 75%.

7. A polypeptide as claimed in Claim 4, 5 or 6 and consisting of said sequence of from 7 to 35 amino acids.

8. A polypeptide as claimed in any preceding claim comprising the amino acid sequence:

Gly Gln Met Gln Pro Val Lys Ala Phe Lys Ile His Asn Lys Ile Trp Val Ile Pro Glu Arg Asp Thr Phe Thr Asn

or an N-terminal fragment thereof comprising up to three additions, substitutions or deletions, the fragment being at least 7 amino acids in length.

9. A polypeptide as claimed in Claim 8 being a fragment consisting of the 7 N-terminal amino acids.

10. A polypeptide as claimed in Claim 8 in which the substitutions are made according to the following table:

Original Amino Acid	Optional Substitute
Arg	His, Lys
Pro	Ala
Ala	Pro
Val	Met, Ile, Ala, Phe
Ile	Met, Leu, Phe, Val
Phe	Met, Tyr, Ile, Leu
His	Gln, Arg, Lys
Gln	Glu, His
Asn	Asp, Gln
Lys	Arg, His
Asp	Asn, Glu
Glu	Gln, Asp
Met	Ile, Leu, Phe, Val

11. A polypeptide as claimed in Claim 10 in which up to two amino acids are substituted, added or deleted.

12. A polypeptide as claimed in Claim 11 in which zero or one amino acids are substituted, added or deleted.

13. A vaccine enhancing agent for use in vaccinating a mammal against botulinum toxin comprising a polypeptide as claimed in any preceeding claim and a pharmaceutically acceptable carrier.

14. A vaccine for vaccinating a mammal against botulinum toxin comprising a polypeptide as claimed in any preceeding claim, a botulinum toxoid vaccine and a pharmaceutically acceptable carrier.

15. A method of vaccinating a mammal against botulism comprising the steps of:

(1) administering to the mammal a vaccine enhancing agent or a vaccine as claimed in Claim 13 or 14, and

(2) subsequently or simultaneously administering to the mammal a vaccine comprising a botulinum toxoid vaccine.

16. A method of vaccinating a mammal against botulism comprising the steps of:-

(1) administering to the mammal a first vaccine comprising a botulinum toxoid vaccine, and

(2) subsequently administering to the mammal a vaccine enhancing agent or vaccine as claimed in Claim 13 or 14.

17. A method as claimed in Claim 15 or 16 in which the toxoid vaccine is pentavalent botulinum (A-E) toxoid vaccine.

18. A polypeptide as claimed in any of Claims 1-12 alone, or in combination with a botulinum toxoid, for use in vaccinating a mammal against botulinum toxin.

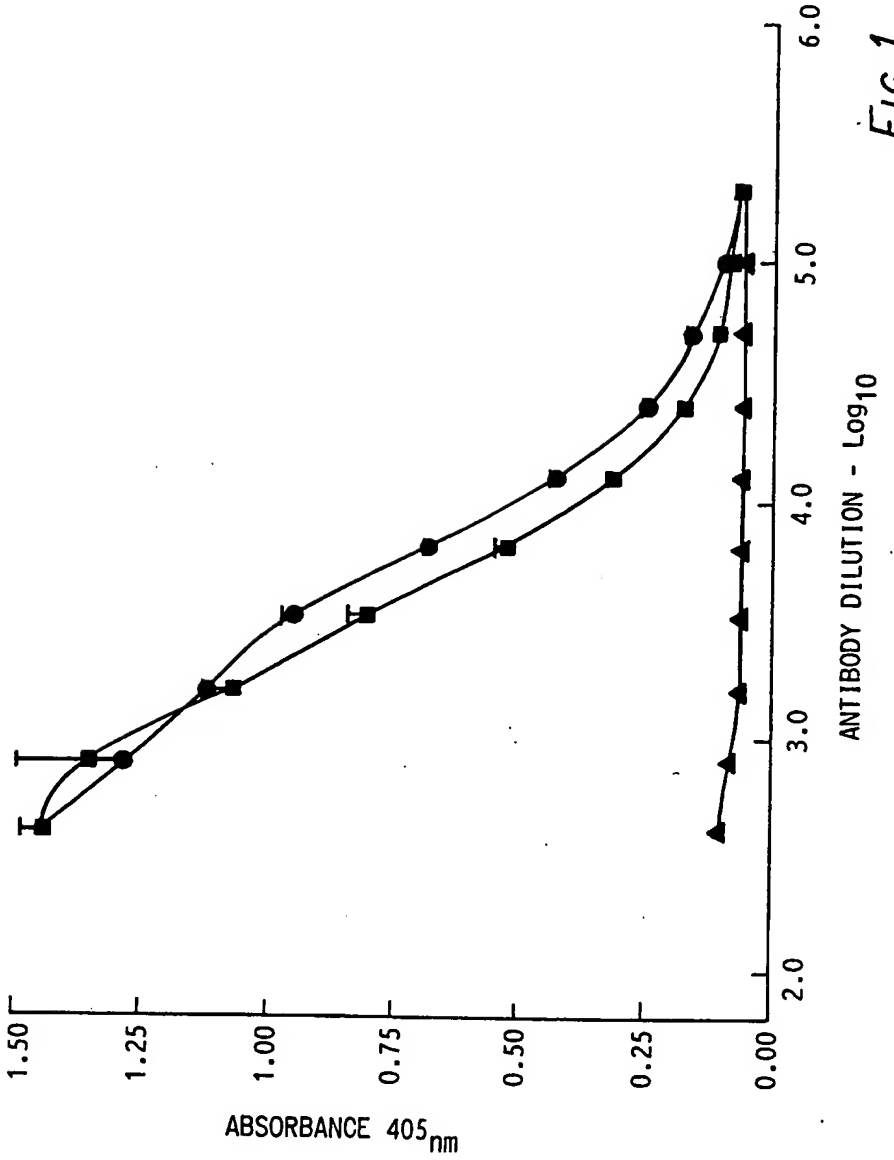
19. A kit for vaccinating a patient against botulinum toxin comprising a supply of a polypeptide as claimed in any of Claims 1-12 and 18 and a supply of a botulinum toxoid vaccine.

20. A method of vaccinating a patient against a disease caused by a polypeptide toxin comprising the steps of:

(1) administering to the patient a vaccine enchancing agent comprising a polypeptide substantially free of toxin activity which contains a T cell epitope and is capable of increasing the anti-toxin antibody titre of a patient subsequently challenged with the toxin, and

(2) subsequently administering chemically de-activated toxin to the patient.

21. Use of a polypeptide as claimed in any of Claims 1-12 in the manufacture of a medicament for vaccinating a mammal against botulism.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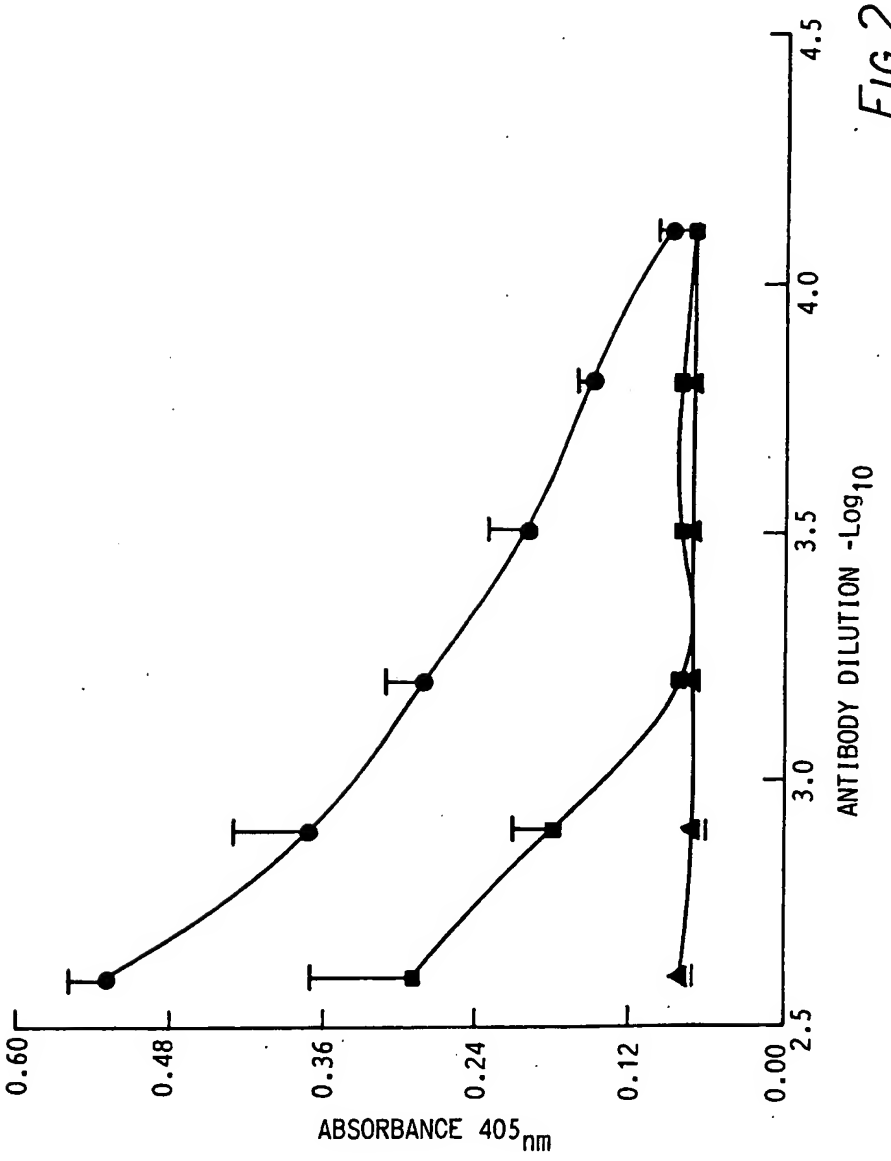
6.0
ANTIBODY DILUTION - Log10

FIG. 1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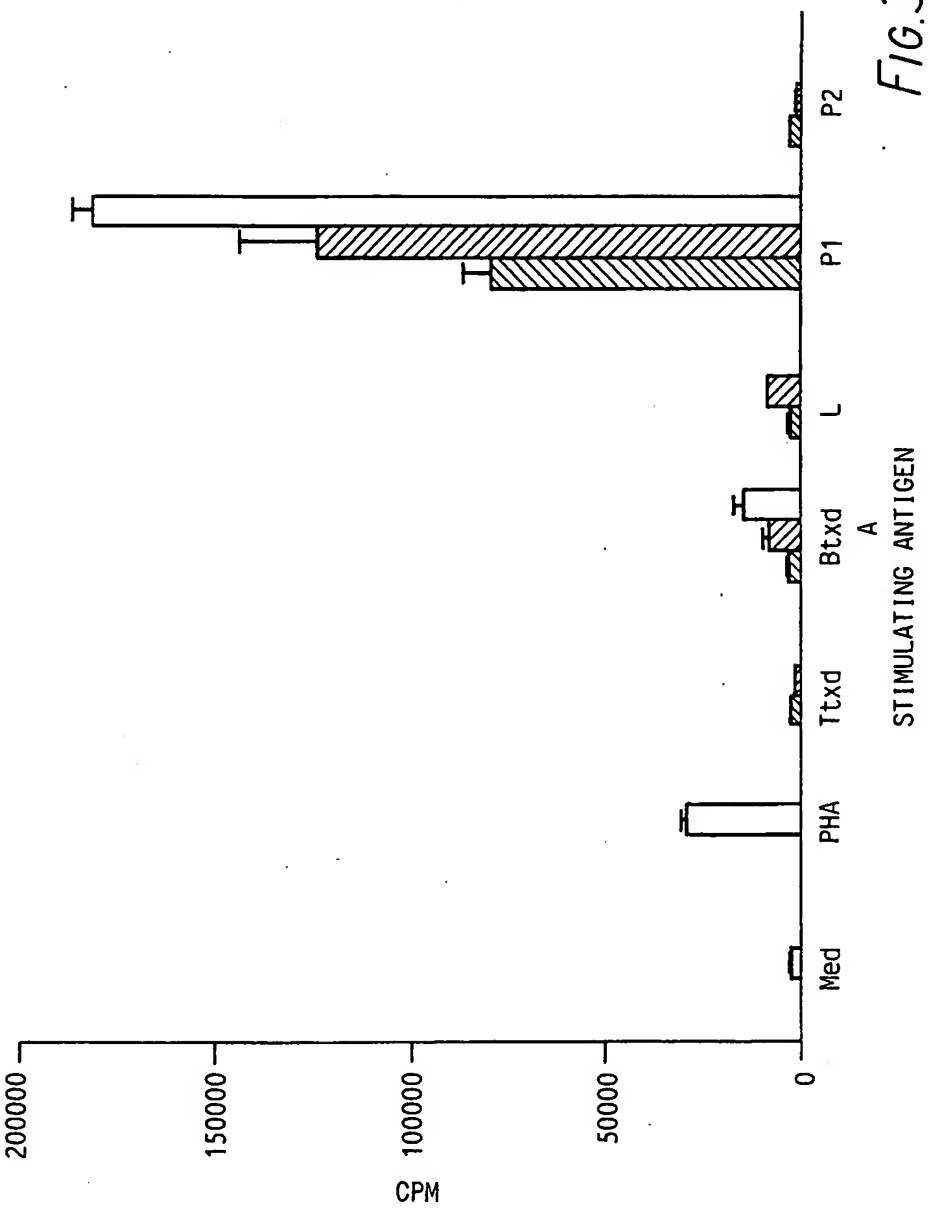
4.5
ANTIBODY DILUTION -Log10

FIG.2


200000
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CPM
Med
PHA
Ttxd
Btxd
A
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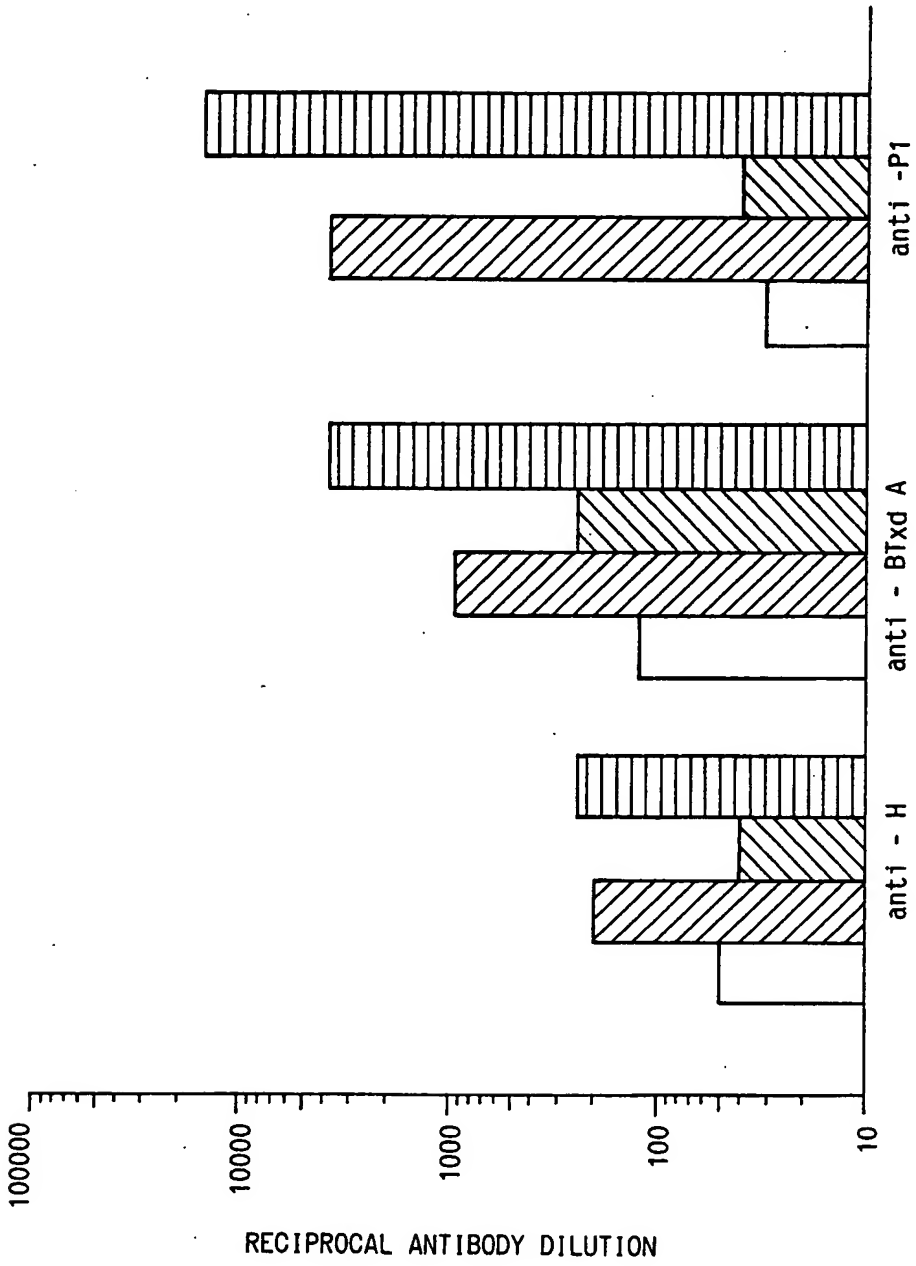
P1
P2
STIMULATING ANTIGEN

FIG.3

FIG.4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anti - BIxd A
anti - H
100000
10000
1000
100
10
RECIPROCAL ANTIBODY DILUTION

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 94/00570

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07K15/04 C07K7/06 C07K7/08 C07K7/10 A61K39/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EUROPEAN JOURNAL OF BIOCHEMISTRY vol. 189, no. 1 , 1 April 1990 pages 73 - 81 THOMPSON ET AL. 'The complete amino acid sequence of the Clostridium botulinum type A neurotoxin, deduced by nucleotide sequence analysis of the encoding gene' see figure 3 ---	8,12
X	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 265, no. 16 , 5 June 1990 , BALTIMORE, MD US pages 9153 - 9158 BINZ ET AL. 'The complete sequence of botulinum neurotoxin type A and comparison with other Clostridial neurotoxins' see figure 2 --- -/--	8,12

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 June 1994	14. 07. 94
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Cupido, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 94/00570

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 267, no. 21 , 25 July 1992 , BALTIMORE US pages 14721 - 14729 KURAZONO ET AL. 'Minimal essential domains specifying toxicity of the light chains of tetanus toxin and botulinum neurotoxin type A' see page 14726, paragraph 2 - page 14727, last paragraph -----	1-21

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB94/00570

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Althoug claims 15-17 and 20 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.